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# Synthesis optimization of lipid-wrapped polymer nanoparticles

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Thesis

# SYNTHESIS OPTIMIZATION OF LIPID-WRAPPED POLYMER NANOPARTICLES

by

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*The darkest moment is before dawn.*

*Hope my sunrise is coming.*

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My parents, thank you for your forever care and support.

**SYNTHESIS OPTIMIZATION OF**

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ABSTRACT

Nanoparticles (NPs) have become a robust drug delivery platform and are in extensive use in the pharmaceutical industry. Lipid-wrapped polymer NPs combine the advantages of liposomes and polymer NPs together and exclude some limitations of both. This thesis reports how to optimize the synthesis outcome of lipid-wrapped polymer NPs by changing the lipid/polymer ratio, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine(DOPS) composition in lipids and the choice of polymer materials. The *in vitro* stability of the synthesized NPs were tested in 1× PBS buffer in terms of size at different time points up to 7-day at room temperature. The lipid-wrapped polymer NPs were prepared by one-step nanoprecipitation and self-assembly aided by bath sonication for five minutes. We characterized NPs by measuring the hydrodynamic diameters and zeta potential through Dynamic Light Scattering (DLS), measuring the absorbance through Ultraviolet–visible spectroscopy (UV-Vis) and observing the morphology and size distributions through Scanning Electron Microscope (SEM). We find the surfactant DOPS ratio has a significant influence on size distribution. And NPs with different polymer materials are stable in 1 × PBS buffer within 7 days. There is a clear negative correlation between PDI and lipid/polymer ratio, which indicates the potential to control PDI by adjusting the lipid/polymer ratio. The result of this project has the potential use in precisely

controlling the sizes and size distributions of drug-loaded NPs and scaling up the production in pharmaceutical manufacture.

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## STATEMENT OF PURPOSE

The focus of this thesis is to tune the physical properties of lipid-wrapped polymer NPs, obtain less than 140 nm sized NPs and narrow NP size distributions. NPs were generated through self-assembly and analyzed by different characterizations. The effects of surfactant DOPS ratio, choice of polymer materials and lipid/polymer ratios will be quantified.

## BACKGROUND

Nanotechnology is playing an important role in the fields of pharmacy and biomaterial [20,37,44]. It has been quickly developing since the 1980s. As the most important participant, NPs have become a robust drug delivery platform and have been in extensive use. Nanocarrier is one of the non-invasive, time-controlled and site-specific approaches for drug delivery in the pharmaceutical industry [13,44]. Different types of nanocarriers, such as micelles, liposomes, dendrimers, polymeric NPs, have been developed to enhance drug delivery efficacy, drug kinetics, and reduce off-target effects[25,44]. Nanocarrier-based chemotherapeutic drugs, either alone or in combination with gene therapies, are currently under development with the goal of improving clinical outcome[1,2,15]. Abraxane®, Doxil®, Genexol®_PM, Myocet®, Depocyt® are successfully marketed products and many others are under clinical trials[35]. Connecting with the pandemic COVID-19, nanocarriers constitute a major part in the vaccine development [45].

Among different sorts of nanocarriers, liposomes are widely studied and have been used to deliver both hydrophilic and hydrophobic drugs in the past decades [5,46], which are spherical lipid vesicles with a bilayer membrane structure consisting of amphiphilic lipid molecules[4,5]. Two most common protocols used in the preparation of liposomes are extrusion and bath sonication, with each method yielding liposomes of different mean sizes and size distributions [24]. Lipid-based carriers show advantages such as cost-effective manufacturing and better drug entrapment efficiency [35]. Liposomes can easily be modified with different surface functional

groups to achieve different features, such as being thermally sensitive for fast drug releasing [5,22] . A few liposome drug formulations have been approved by the Food and Drug Administration (FDA) and are commercially available [1,5,12,20,21]. Liposomal formulations not only have benefits[5], but also limitations such as relatively complicated fabrication steps, low loading efficiency for hydrophobic drugs, burst release kinetics of encapsulated drugs, instability during storage leading to short shelf-time, and limited applicable drug delivery[31].

Another category of nanocarriers, biodegradable polymeric NPs, also called for attention due to their bigger capacity of drug loading than liposomes, smaller particle size and narrower size distribution[35], tissue penetrating ability, a greater variety of preparation methods, availability of various polymers, improved stability in biological fluids, versatile drug loading and release profiles[23,29,43]. They can also be encapsulated into extracellular vesicles as therapeutic entities[43]. Most polymeric NPs such as chitosan-based NPs and PLGA-based NPs are internalized into the cells through a mechanism called endocytosis, and are then transported into lysosomes for degradation [1, 41]. However, polymer NPs have not yet obtained as much translational success as liposomes [5]. Clinical applications of multifunctional polymer-based systems are still scarce[20] due to use of toxic organic solvents in the production process, poor drug encapsulation for hydrophilic drugs, drug leakage before reaching target tissues, polymer cytotoxicity, polymer degradation, and scale-up issues[17,23,31].

Based on the intrinsic limitations of both liposomes and polymeric NPs, lipid-wrapped polymer NPs have attracted interests as a delivery platform. The biomimetic

characteristics of lipids and architectural advantage of polymer core are combined to yield a theoretically superior delivery system, while avoiding some of the limitations of the building blocks [3,5,23,35]. This type of hybrid NP has high drug encapsulation yield, tunable and sustained drug release profile, excellent serum stability, and potential for differential targeting of cells or tissues [3]. Precise control over the drug quantity and the release rate in specific parts of the body has numerous advantages over conventional drug release, such as enhancing bio-availability and minimizing deleterious side effects. These benefits are even more important for patients suffering from certain diseases that require multiple dosage regimes, such as chronic diseases because they can improve patients' quality of life [20]. Drugs may be incorporated into NPs by different methods. Regarding the potential use of NPs in this project, drugs are usually incorporated within the NPs during the course of the preparation of NPs [45].

The typical structure of the lipid-wrapped polymer NP is comprised of the following components: (i) a hydrophobic polymeric core where poorly water-soluble drugs are incorporated with high loading yields; (ii) a lipid layer surrounding the core that acts as a highly biocompatible shell and as a molecular fence to promote drug retention inside the polymeric core; (iii) a hydrophilic polymer stealth layer outside the lipid shell to enhance NP stability and systematic circulation lifetime, which is usually the lipid-PEG(Polyethylene glycol) conjugates[5,23] as illustrated in figure 1[5]. PEG provides stealth effects against uptake by the immune system[23] and increases the mean residence time in circulation in addition to steric stabilization[35]. Numerous

studies are ongoing to develop stealth coatings for the NPs such that opsonin protein adsorption to the NPs and phagocytosis by reticulo-endothelial system (RES, also called the MPS, mononuclear phagocyte system) are inhibited [45]. Both PEG chain length and lipid/lipid-PEG molar ratio have significant impact on NP stability[5]. Besides, surfactants are customarily utilized during NP synthesis to prevent aggregation despite their numerous disadvantages[19].

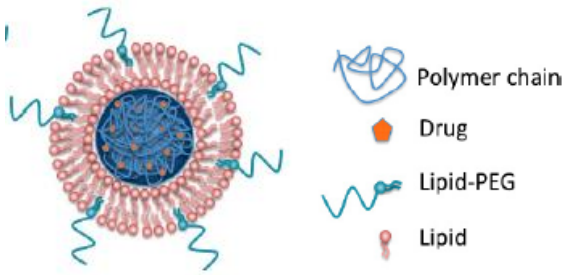


Figure 1 The typical structure of lipid-wrapped polymer NP

There are generally two methods to synthesize the lipid-wrapped polymer NPs [3,5,23,32]. One method requires two steps. The polymeric cores and liposomes are prepared separately using two independent processes; then the two components are combined by direct hydration, sonication, or extrusion to obtain the desired lipid shell–polymer core structure[23]. The outcome of this method is lipid bi-layer wrapping a polymeric core as seen in figure 1.

The other method requires only one step: a nanoprecipitation process is synchronized with a simultaneous self-assembly process [23]. In this method, there are

two different ways of mixing the organic phase and aqueous phase. One way is a modification of the emulsion/solvent evaporation method first reported by Gurny et al.[23,28]. The polymer and drugs are dissolved in a water-immiscible organic solvent. A predetermined amount of lipid is dispersed in water by bath sonication, mechanical stirring or heat. The organic phase mixes with aqueous phase, then the mixture is sonicated by probe sonicator in an ice bath. The organic phase will disperse into tiny nanodroplets, which are nanospheres with lipid layer coats[23].

In another way utilized in this project, polymer solution is added dropwise into the lipid solution. Then external energy is used to expedite the synthesis. The outcome is lipid monolayer wrapping a polymeric core as in figure 2[5].

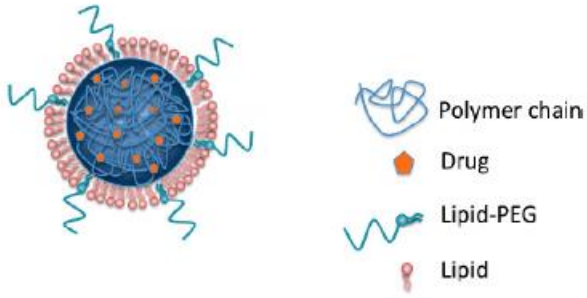


Figure 2 The structure of lipid monolayer NP product synthesized by self-assembly

The physical properties and drug encapsulation characteristics of the NPs were controlled and optimized by varying the synthesis parameters[19]. The advantages of the one-step method are faster synthesis[23], simpler steps (namely less

possible errors in operation), easier future scale-up[3,9] and more effective cost[5], etc. A major advantage of nanoprecipitation is that stabilizing surfactants commonly added during NP synthesis are not required to prevent NP aggregation [19]. Many researchers use the nanoprecipitation method to synthesize surface-modified polymer NPs as drug carriers, i.e. Trimethylated chitosan (TMC) surface-modified NP for brain drug delivery[9]. In the process, the lipid and polymer contact is favored by electrostatic interactions, hydrophobic attractions, or van der Waals forces[23].

Speaking of the polymeric material choice, Poly (lactide-co-glycolide) (PLGA) is a FDA approved biodegradable polymer which has been widely used as a model biomaterial for sustained, targeted NP synthesis[1,36] due to PLGA's clinical biocompatibility, tunable biodegradation and resorbable by-products[19,27]. PLGA-based NPs are applicable for both hydrophilic and hydrophobic drugs, and biological therapeutic macromolecules including peptide and protein[1,30]. And PLGA NPs with drugs are preferable to traditional oral, intramuscular or intravenous therapies because they flow through capillaries, protect molecules from enzymatic degradation, enable delivery of non-traditional molecules, are modifiable for receptor targeting and can be internalized by cells for cytoplasmic drug delivery [14,16,19]. Furthermore, they solubilize hydrophobic drug molecules by encapsulating and separating drugs from aqueous physiological environments[19].

Besides PLGA, Poly-D,L-lactic-acid (PLA) has been extensively used for its biocompatibility and biodegradability[38,45]. It is particularly suitable for applications where a slow release of bioactive agents *in situ* is required [38,42]. The numerous PLAs

available in the market can be differentiated by molecular weight, degree of crystallinity, inherent viscosity and proportion of D and L isomers [38]. Nanospheres and NPs of PLA and PLGA are generally described as having characteristic dimensions smaller than 10 microns in diameter and thus are available for direct endocytosis or phagocytosis into leukocytes, monocytes, macrophages and other cells of the reticulo-endothelial system. Studies to date indicate that PLA and PLGA microspheres containing bioactive agents are biocompatible, including when used in therapeutic applications *in vivo*, and they did not exhibit untoward reactions either locally or systemically[45].

The NP structure design in this study was inspired by the size and lipid composition of human immunodeficiency virus type 1(HIV-1), in which cholesterol and phospholipids keep a certain ratio. The composition of lipid shell in this project is 40% Cholesterol, 60% in total of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine(DPPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine(DOPS, sodium salt). In order to characterize NPs, two different fluorescent dyes are added separately in the lipid membranes and polymeric cores of some NPs. Surface moiety modification of PLGA-based NPs could greatly enhance the interaction between NPs and biological cells, resulting in longer half-life, better cellular uptake and targeting effects.

In this project, drugs were not actually loaded in the NPs. The NPs were not designed for *in vivo* experiments, therefore PEG was not added in the lipids. The structure of lipid-wrapped polymer NPs in this project contains simply the lipid

monolayer shell and polymeric core (Figure 3). NP diameters under 150nm were desired in this project because smaller NPs are more easily engulfed by cells [6,19, 34] for intracellular drug delivery [18,19,33]. Narrowly unimodal distributed NPs were desired in order to minimize complications in filtering, purification and future characterization of cellular interactions[19,39]. The NP products were purified right after synthesis by three washes. Centrifugation sometimes causes NPs to agglomerate into unacceptably large clusters[19,39]. However, purification is critical for cellular drug delivery studies because potential therapeutic effects must be attributed to drug-loaded NPs[19]. Noteworthily, a novel, nondestructive purification protocol involving transient sodium dodecyl sulfate (SDS) adsorption to NPs followed by centrifugation and dialysis was claimed to be able to prevent centrifugation-induced surfactant-free NP aggregation [19].

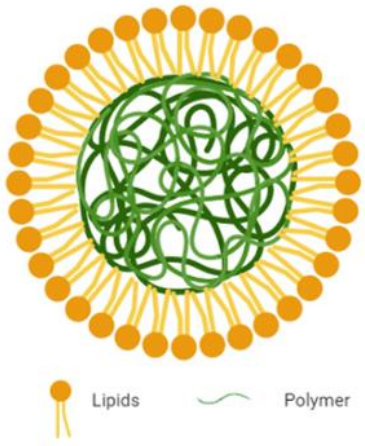


Figure 3 Structure of lipid-wrapped polymer NPs in this project

Nowadays we have dozens of advanced technologies to characterize NPs. Most research uses Dynamic Light Scattering (DLS) to measure the hydrodynamic diameters (Z-ave), polydispersity index (PDI) and zeta potentials of NP, given its fast and convenient way to operate. PDI can provide additional information about the level of agglomeration in solution [8]. Ultraviolet–visible spectroscopy (UV-Vis) can be used to measure the absorbance. To obtain more precise results and satisfy different characterization needs, sensitive and multiplex optical imaging techniques are needed[7]. Scanning electron microscopy (SEM) is usually applied to measure the physical dimension of the particles, visualize the size, size distribution, and morphology of NPs[5,23,32]. Conventional fluorescence microscopy can prove the successful combination of lipids and polymers. Besides, the internal core-shell structure and lipid shell thickness are typically measured by transmission electron microscopy (TEM) [5, 23].

Here we report a clear negative correlation between PDI and lipid/polymer ratio on lipid-wrapped PLGA NPs. It indicates the potential for us to control and predict the PDI by adjusting the lipid/polymer ratio. The condition combination of more than 4 μmol/mg lipid/polymer ratio and less than 1 mol% of surfactant DOPS easily causes aggregation, significantly increases PDI, and results in experiment failures. The choice of PLA or PLGA as polymer material doesn't significantly affect the short-term *in vitro* stability in 1× PBS buffer of lipid-wrapped polymer NPs with 3%DOPS and 6 μmol/mg lipid/polymer ratio.

# MATERIALS AND METHODS

## 1. Materials

Poly(D,L-lactide) high molecular weight(PLA HMW), ester terminated, R207S, MW 209,000[38] and Poly (D,L-lactide-co-glycolide) Lactide:Glycolide(PLGA) 50:50, ester terminated, RG503, MW 24,000-38,000 were obtained from Sigma-Aldrich (St Louis, MO).

Cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine(DPPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine(DOPS, sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL).

Other reagents including anhydrous ethanol, Milli-Q water, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) powder, NaCl powder , acetonitrile, Poly-L-lysine(0.1% (w/v) in $H_2O$) and 10× Phosphate-buffered saline(PBS) buffer were obtained from Sigma-Aldrich (St Louis, MO). Micro slides for SEM images were purchased from Corning Incorporated (Corning, NY).

## 2. Liposome preparation

Cholesterol, DPPC and DOPS with specified mol% were mixed in a round-bottom flask. The total amounts of lipids were 0, 1, 2, 4, and 6 μmol. After evaporating the organic solvent (chloroform) using Rotavapor (BüCHI, R-200, Brinkmann Instruments, Westbury, NY) at 30 ℃ for 10 min, a thin uniform lipid layer formed on the bottom. The flask was then put on vacuum overnight. Next, 1.2 mL of 20 mM HEPES buffer was added into the flask, forming a cloudy solution after vigorous

agitation. Under Argon protection, liposomes were made of the lipid mixture by probe sonication (120 Sonic Dismembrator, Fisher Scientific, Waltham, MA).

## 3. Lipid-wrapped Polymer NP synthesis

The prepared liposome solution was mixed with 4 wt% ethanol aqueous solution for 15 minutes. The polymers were dissolved in acetonitrile to make a solution with a concentration of 2.5 mg/mL. The polymer solution was then added into the liposome solution dropwise. The volume ratio of organic phase/aqueous phase in the mixture is always 1/10. To synthesize the lipid-wrapped polymer NPs, the mixture was sonicated in a bath sonicator for 5 min under room temperature (Branson Ultrasonics 5510, Danbury, CT). The lipid-wrapped polymer NPs were washed 3 times (15 min per washing cycle) to remove organic solvent and free lipids using Amicon Ultra-4 centrifugal filter with molecular weight cutoff 10 kDa (Millipore Sigma, Burlington, MA).

## 4. Characterization

### *4.1 Size distribution and zeta potential measurements*

The hydrodynamic diameters (Z-ave) and polydispersity (PDI) of liposomes and the lipid-wrapped polymer NPs after synthesis (before and after washing) were measured by dynamic light scattering (DLS) in Zetasizer (ZEN3690, Malvern, Worcestershire, UK). All samples for size measurements were measured in triplicate

with Milli-Q water. Liposomes were diluted 10 times in water for size measurements. Lipid-wrapped polymer NPs were measured without dilution before washing, and were diluted 10 times in water for measurements after washing. The zeta potentials of the lipid-wrapped polymer NPs after washing were measured in 10 mM NaCl solution (Zetasizer).

### *4.2 Morphology and size*

Samples for SEM were incubated on the Poly-L-lysine-treated microscope cover glass substrates for 20 min. The glass substrates were washed by water drops three times. Argon flowed around until the glass substrates seemed dry. Samples were then put in the fume hood for overnight drying. All samples were Au-sputtered before being subjected to imaging. SEM images were generated on a Zeiss Supra 40 VP SEM with 5 eV EHT.

### *4.3 Absorbance measurements*

Samples were diluted 8 times in Milli-Q water. The absorbance measurements at $\lambda$=500 nm of selected samples were completed on the Spectronic 200 UV-vis spectrometer (Fisher Scientific, Waltham, MA).

### *4.4 Stability measurements*

To monitor the stability of lipid-wrapped polymer NPs, the particle sizes and PDI were measured using a Zetasizer at different incubation time points, including 0 min (right after the mixture of NP and PBS), 10 min, 30 min, 1h, 24h and 7-day. The NPs were incubated in 1 × PBS buffer (pH 7.4) at room temperature. Stock solution for incubation was made by diluting the product 10 times in 1 × PBS buffer. The samples for DLS measurements were made by diluting the stock solution 10 times in water.

## 5 Experiment design

In this project, three independent variables were explored: lipid/polymer ratio, DOPS ratio and the choice of core polymer materials. The incipient parameters are 1 μmol lipids with different ratios of DOPS and different 1mg core polymer materials. Then the scale and gradient of each parameters were revised after a few explorations.

| Lipids / %DOPS | 1μmol | 2μmol | 4μmol | 6μmol |
|---|---|---|---|---|
| 0 | √ | | | |
| 1 | √□≈ | | | √⋆ |
| 3 | √≈ | √≈ | √≈ | √≈ |
| 10 | √≈ | | | √ |

Table 1 Experiment summary of PLA HMW NPs

| Lipids / %DOPS | 0(core only) | 1μmol | 2μmol | 3 μmol | 4μmol | 6μmol |
|---|---|---|---|---|---|---|
| 0 | √ | √ | √ | | √⋆ | √⋆ |
| 1 | | √≈ | | | | √⋆ |
| 3 | √ | √≈ | √ | | √ | √ |
| 6 | | √□ | √≈ | √≈ | √≈ | √□ |
| 10 | √ | √ | | | | √ |

Table 2 Experiment summary of PLGA NPs

Meanings of symbols:

√, accomplished experiments

⋆, failed experiments

□, samples were characterized by SEM

≈, the absorbance of diluted samples was measured by UV-Vis

## RESULTS AND DISCUSSION

### 1. Morphology and size (SEM images)

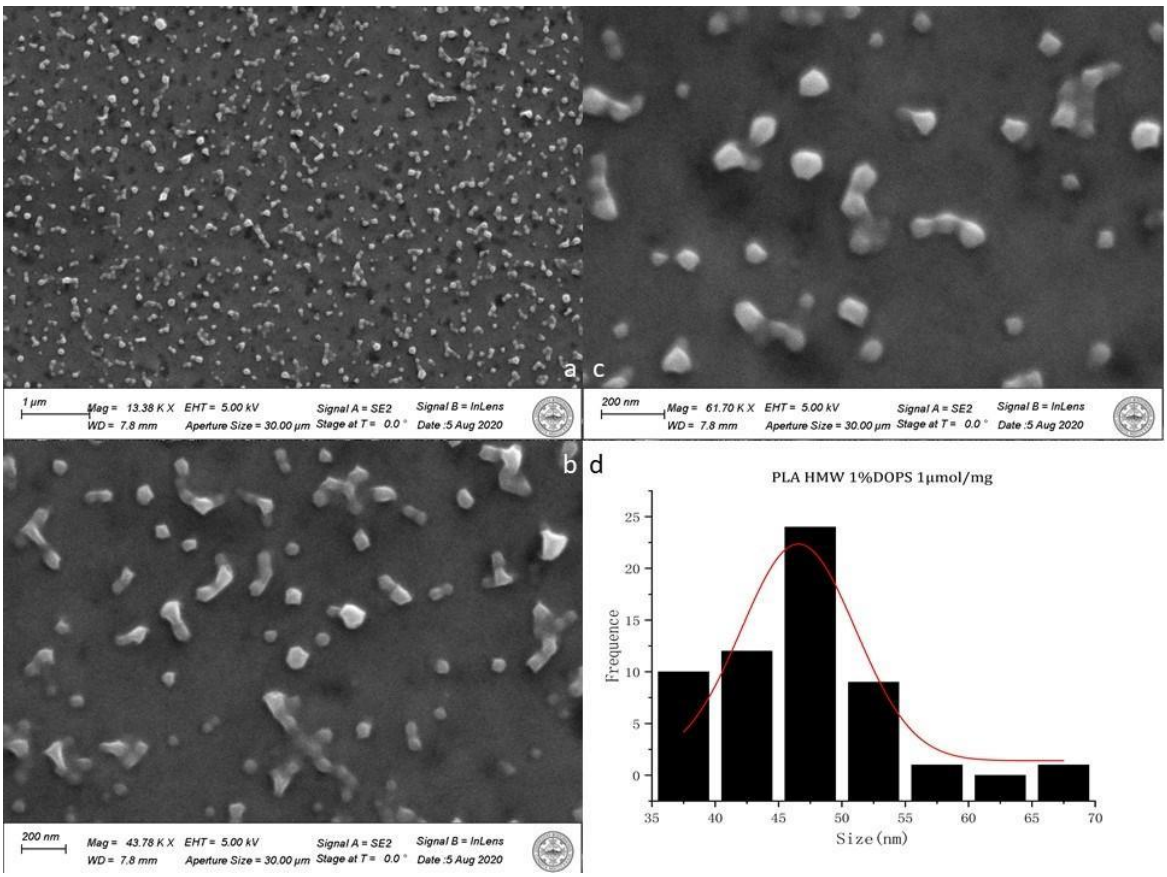


Figure 4 SEM images and size distribution analysis of PLA HMW NPs with 1%DOPS and 1 µmol/mg lipid/polymer ratio. (a) (b) (c) are SEM images. Particles have mean size 45.89nm and standard deviation (SD) 5.54nm; PDI=0.12 (SEM Images in this project were processed with ImageJ.) (d) is the size distribution analysis.

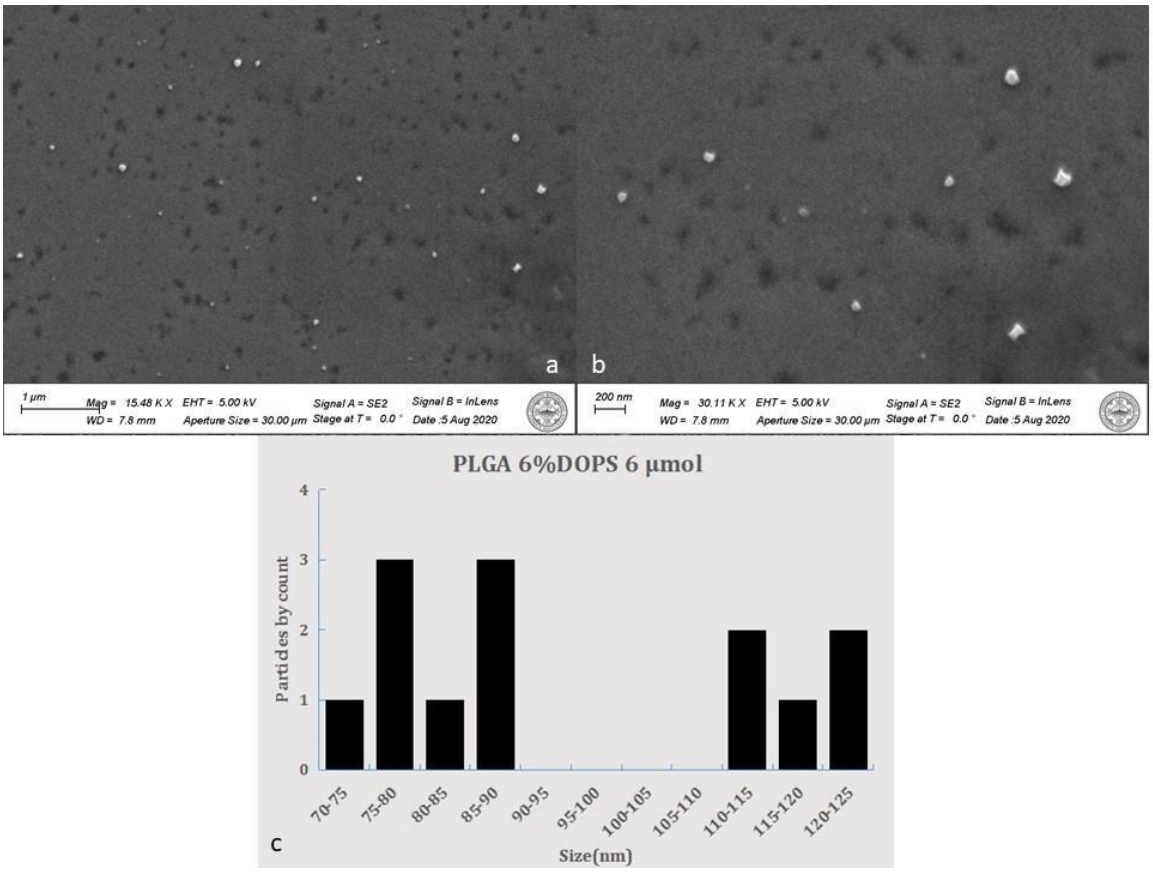


Figure 5 SEM images and size distribution analysis of PLGA NPs with 6%DOPS and 6 µmol/mg lipid/polymer ratio. (a) (b) are SEM images. Particle size: 95.07±18.58nm, PDI=0.20 (c) is the size distribution analysis. Samples had a low concentration so bimodal distribution was observed.

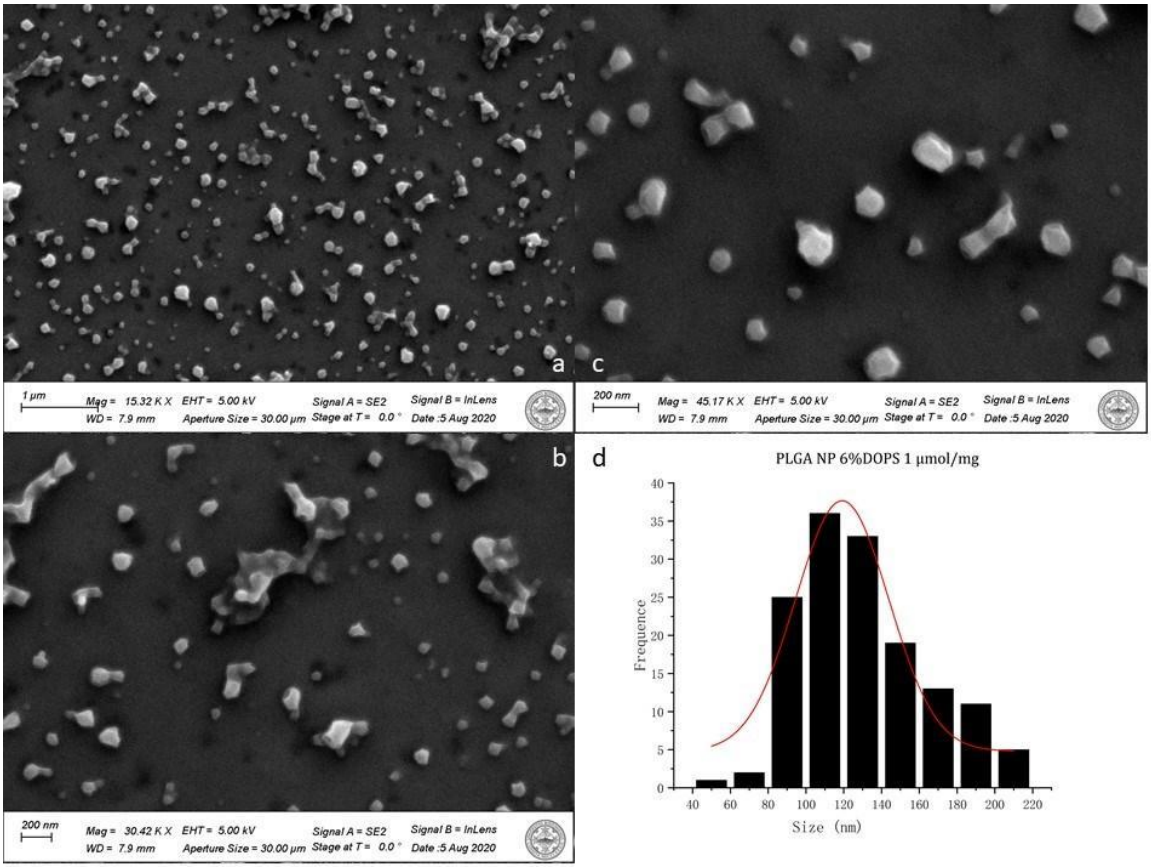


Figure 6 SEM images and size distribution analysis of PLGA NPs with 6%DOPS and 1 µmol/mg lipid/polymer ratio. (a) (b) (c) are SEM images. Particle sizes 131.7±32.9nm; PDI=0.25 (d) is the size distribution analysis.

## 2. Absorbance measurements

λ=500nm Dilution factor: 8

| Sample composition | 1%DOPS 1 μmol/mg | 3%DOPS 1 μmol/mg | 10%DOPS 1 μmol/mg | 3%DOPS 2 μmol/mg | 3%DOPS 4 μmol/mg | 3%DOPS 6 μmol/mg |
|---|---|---|---|---|---|---|
| Absorbance | 0.24 | 0.27 | 0.15 | 0.15 | 0.05 | 0.07 |

Table 3 Absorbance of lipid-wrapped PLA NPs (diluted 8 times)

| Sample composition | 1%DOPS 1 μmol/mg | 3%DOPS 1 μmol/mg | 6%DOPS 2 μmol/mg | 6%DOPS 3μmol/mg | 6%DOPS 4 μmol/mg |
|---|---|---|---|---|---|
| Absorbance | 0.04 | 0.12 | 0.03 | 0.06 | 0.09 |

Table 4 Absorbance of lipid-wrapped PLGA NPs (diluted 8 times)

## 3. The effects of surfactant (DOPS)

Surfactant DOPS [11] was added to provide negative charges on the outside surface, in order to prevent aggregation. But some NPs aggregated during the washing steps. The reasons should be insufficient surface charges or inappropriate lipid-polymer ratios. In Table 1 and 2, the failures happened when ≤1% DOPS was added in the samples with ≥ 4 μmol/mg lipid/polymer ratio. Therefore, NPs tended to gather and aggregate in centrifugation without DOPS. In the later stage of experiment design, the 0%DOPS condition was not in consideration.

## 4. PLA HMW & PLGA as core material

The *in vitro* stabilities of PLA HMW and PLGA products were compared in Figure 4. They were synthesized under the same conditions with 3%DOPS and 6μmol lipids. No colloids were observed. Given the common use of both polymers in

industry, both polymers are stable in 1 ×PBS buffer in short terms (up to 7-day). Noteworthily, the PDI of both NPs kept increasing with time.

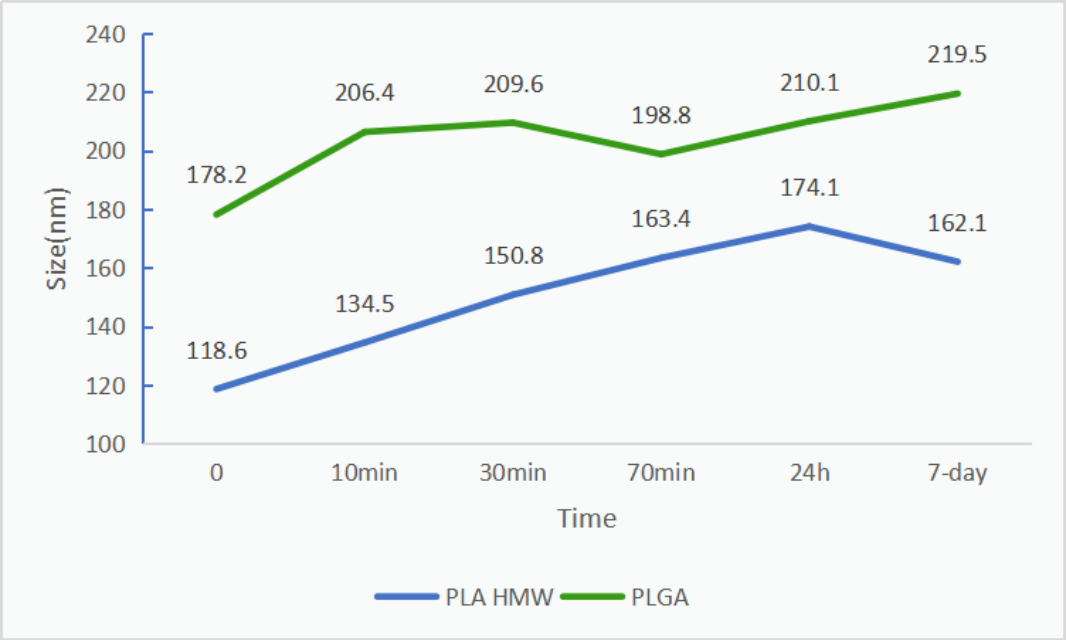


Figure 7 *In vitro* stability comparison of PLA HMW NPs and PLGA NPs, with 3%DOPS and 6 μmol/mg (lipid/polymer ratio) in 1 × PBS buffer

## 5. Lipid/polymer ratio

As mentioned in the experiment design section, the incipient ratio of lipid and polymer was 1 μmol/mg. When the ratio of lipid/polymer increased to 4μmol lipid/ 1mg polymer or higher, failures began happening on products with ≤ 1mol% DOPS.

When analyzing PLGA NPs with 3%DOPS, there is no clear linear positive or negative relationship between lipid/polymer ratio and particle sizes.

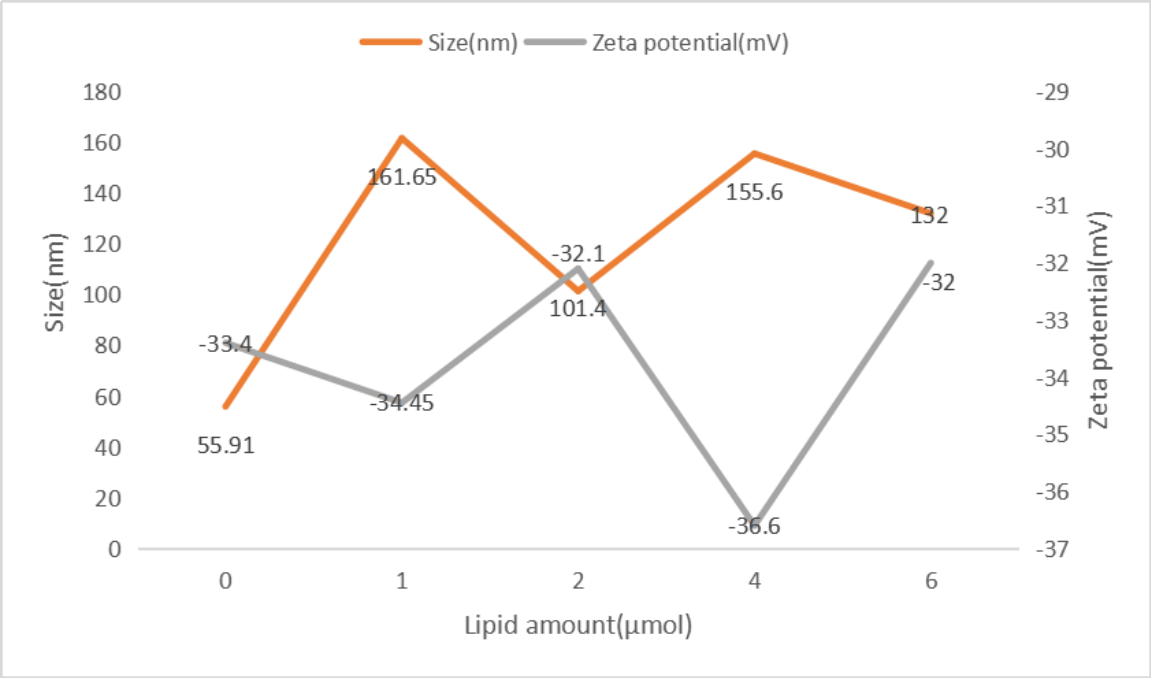


Figure 8 Size and zeta potential of 3%DOPS-PLGA NPs

However, the PDIs of PLGA NPs with 3% DOPS decreased as the lipid/polymer ratio increased. The clear negative correlation between PDI and lipid/polymer ratio shows a potential of controlling and even predicting the PDI by adjusting the lipid/polymer ratio. The point of 0 lipid/polymer ratio is a control.

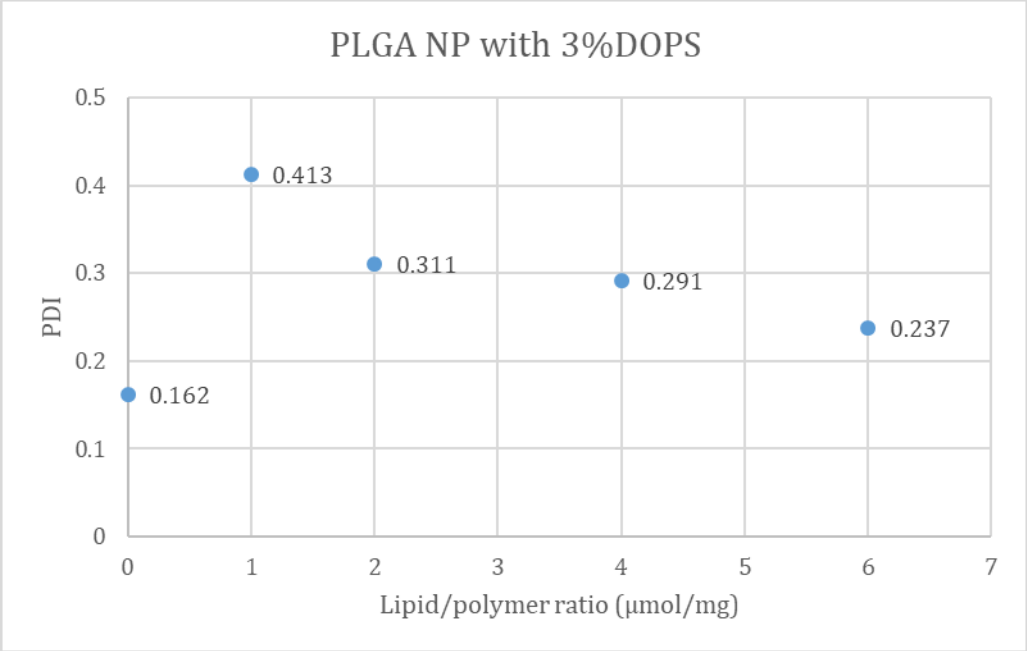


Figure 9 PDIs of PLGA NPs with 3% DOPS and different lipid/polymer ratios

## SUMMARY

The choice of PLA or PLGA as polymer material doesn't significantly affect the short-term *in vitro* stability of lipid-wrapped polymer NPs in 1 × PBS buffer. Its effect on size is to be explored further. The condition combination of ≥ 4 μmol/mg lipid/polymer ratio and ≤ 1 mol% of surfactant DOPS easily causes aggregation, significantly increases PDI, and results in experiment failures. PDI was found to hold a negative correlation with the lipid/polymer ratio (up to 6 μmol/mg) on lipid-wrapped PLGA NPs. It indicates a potential to control and even predict PDI by properly adjusting the lipid/polymer ratio.

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**CURRICULUM VITAE**